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Coinfection and Emergence of Rifamycin Resistance during a Recurrent *Clostridium difficile* Infection

Stevenson, Emma; Kuehne, Sarah; Major, Giles; Spiller, Robin; Minton, Nigel P

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1 **Co-infection and emergence of rifamycin resistance during a recurrent**
2 ***Clostridium difficile* infection**

3 ^{1,2}Emma C. Stevenson, ³Giles A. Major, ³Robin C. Spiller, ^{1,2}Sarah A. Kuehne, and
4 ^{1,2}Nigel P. Minton[#].

5 ¹ Nottingham BBSRC/EPSRC Synthetic Biology Research Centre, Clostridia Research
6 Group, School of Life Sciences, the University of Nottingham, Nottingham, UK

7 ² NIHR Biomedical Research Unit in Gastrointestinal and Liver Diseases at Nottingham
8 University Hospitals NHS Trust and The University of Nottingham

9 ³ QMC West Block E Floor, Queen's Medical Centre, Nottingham, NG7 2UH, UK

10 **Corresponding author:** Minton, N.P. (nigel.minton@nottingham.ac.uk), Nottingham
11 BBSRC/EPSRC Synthetic Biology Research Centre, Clostridia Research Group, School of
12 Life Sciences, the University of Nottingham, Nottingham, UK. Tel. +44 (0)115 84 67458

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14 **Running Title:** Recurrent *C. difficile* infection

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Emma Stevenson: Biosciences, College of Life and Environmental Sciences, 4th floor, Geoffrey
Pope. University of Exeter, Stocker Road, Exeter, EX4 4QD, UK

20 **Abstract**

21 *Clostridium difficile* (*Peptoclostridium difficile*) is a common health care associated infection
22 with a disproportionately high incidence in elderly patients. Disease symptoms range from
23 mild diarrhoea through to life threatening pseudomembranous colitis. Around 20% of patients
24 may suffer recurrent disease which often requires re-hospitalisation of patients.

25 *C. difficile* was isolated from stool samples from a patient with two recurrent *C. difficile*
26 infections. PCR-ribotyping, whole genome sequencing and phenotypic assays were used to
27 characterise these isolates.

28 Genotypic and phenotypic screening of *C. difficile* isolates revealed multiple PCR-ribotypes
29 present, and the emergence of rifamycin resistance during the infection cycle.

30 Understanding both the clinical and bacterial factors that contribute to the course of recurrent
31 infection could inform strategies to reduce recurrence.

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40 Introduction

41 *Clostridium difficile* (*Peptoclostridium difficile*) is a common health care associated infection
42 with a disproportionately high incidence in elderly patients. Recurrent *Clostridium difficile*
43 infection (CDI) is known to occur in approximately 20% of patients following withdrawal of
44 treatment antibiotics (1), however, this may rise to 65% if a patient has a prior history of CDI
45 (2). Recurring CDI not only causes distress to patients, but is also a substantial burden on the
46 healthcare system due to the increased cost (3) associated with possible prolonged stay or re-
47 admission of a patient to hospital and the re-administration of diagnostic tests and antibiotics
48 (4). Collaborations between clinicians and researchers are contributing to the knowledge of
49 how both the host and bacterium are affecting recurrent disease, in order to reduce recurrence
50 rates, through personalised patient care regimens (5).

51 In this study multiple stool samples from a patient enrolled in a clinical trial (see case
52 information) were collected and *C. difficile* was isolated. The strains isolated from these
53 samples were then phenotypically and genotypically characterised to deduce if changes in the
54 *C. difficile* strain genotype and phenotype could have contributed to recurrent infection.

55 Case

56 A male participant (01008) aged 85 years who had a history of chronic kidney disease and
57 was on multiple long term medication including, bumetanide, candesartan, digoxin,
58 simvastatin, doxazosin, ferrous fumarate and prednisolone. Three months prior to trial
59 enrolment the participant received cefuroxime and gentamicin for a urinary tract infection.
60 One month prior to trial enrolment he was admitted to the hospital, where he received
61 clindamycin (both orally and intravenously) for an infected leg ulcer.

62 In June 2013, after being re-admitted to hospital, he was diagnosed with CDI (Table 1;
63 sample A), was treated with metronidazole (MET) and discharged. He was diagnosed again
64 with CDI in July (Table 1; sample B) and was again treated with MET.

65 In July 2013, within 5 days of cessation of successful treatment of CDI with MET (defined as
66 cessation of diarrhoea for 2 or more days with no loose stools, assessed), he was enrolled on a
67 randomised double blind placebo controlled clinical research trial, aimed at using Rifaximin
68 for reducing relapse of *Clostridium* associated diarrhoea (RAPID- NCT01670149). A
69 simplified study time line of stool sample collection is outlined in Figure 1.

70 The participant successfully provided one trial stool sample (Table 1; sample C). However
71 within 10 days, suffered another diarrhoeal episode (Table 1; sample D), which was initially
72 toxin negative as confirmed using the C. DIFF QUIK CHEK complete kit (Alere) and PCR
73 using the BD MAX™ Cdiff kit (BD Molecular Diagnostics). However, 6 days after sample D
74 he provided another sample (Table 1; sample E) which was toxin positive and therefore
75 defined as relapse according to the RAPID trial protocol (onset of >3 loose bowel movements
76 per day for at least 2 consecutive days combined with a positive toxin assay (with or without
77 a positive *C. difficile* culture)). He was treated with MET and over 8-10 weeks provided two
78 more trial samples (Table 1; samples F & G). Participant notes revealed that during the period
79 when he provided more RAPID samples, he was again diagnosed with CDI (September) and
80 treated with Vancomycin (VAN).

81 The participant was again admitted to hospital and treated with tazocin and subsequently
82 suffered another CDI (Table 1; sample H), around his time of death, in January 2014 due to
83 multiple organ failure. As this was a randomised double blind placebo controlled clinical
84 research trial, it was not known if participant 01008 was on placebo or rifaximin therapy, at
85 the point at which this research was undertaken.

86 **Methods**

87 A total of eight stool samples were collected from the participant (Table 1). These included
88 diagnostic specimens from the Queens Medical Centre (QMC) microbiology department,
89 Nottingham, that were proven *C. difficile* toxin positive by using the *C. diff* Quik Chek
90 complete kit (Alere) and PCR using the BD MAX™ Cdiff kit (BD Molecular Diagnostics).
91 *C. difficile* was cultured from ~ 300mg of stool using a previously published protocol (6). 1-
92 20 *C. difficile* colonies were isolated per sample (Table 2).

93 ***C. difficile* typing**

94 After 48 hours (hrs) of growth on Cefoxitin Cycloserine Egg Yolk (CCEY) (6) agar, up to 20
95 individual *C. difficile* colonies from each stool specimen were inoculated into a single well of
96 a 96-well plate containing 200 µL anaerobic Brain Heart Infusion (Oxoid) plus 0.1% L-
97 cysteine (Sigma) (BHIS) broth, leaving one well blank as a control. The plate was sealed
98 with a breathable sterile film and incubated for 24 hrs in an anaerobic workstation (Don
99 Whitley) (CO₂:H₂:N₂ (80:10:10 vol:vol:vol.). After 24 hrs the wells were checked for
100 turbidity. Overnight cultures were then diluted 10-fold with sterile PCR grade water into a
101 fresh 96-well plate, sealed with film and stored at -20°C until required. One drop of 100%
102 glycerol was added to the BHIS cultures and the plate was stored at -80°C until required.
103 Every colony that was isolated from each stool sample was subjected to in-house ribotyping
104 with the diluted cultures as mentioned above. PCR amplification of the 16s rRNA intergenic
105 spacer region was carried out according to a modified protocol obtained from the former *C.*
106 *difficile* ribotyping laboratory in Cardiff (Supplementary data). PCR-ribotype profiles were
107 analysed with a QIAxcel capillary Electrophoresis machine (Qiagen) using the OL400
108 programme with the QX 15bp-1Kb alignment marker and the QX Size Marker 50 bp –800
109 bp. Individual profiles were assessed, and then one isolate from each distinct typing profile

110 that had been obtained from each stool sample, was re-cultured onto BHIS-CC agar and
111 stored as a glycerol stock.

112 These stock strains were sent for official ribotyping via the *C. difficile* ribotyping network
113 (CDRN) service in Leeds and used for downstream characterisation.

114 **Phenotypic Characterisation**

115 Growth, sporulation and toxin A and B ELISAs were performed on all isolates.
116 (Supplementary data)

117 **Antibiotic Susceptibility**

118 Isolated strains were tested for antibiotic resistance to metronidazole (MET) and vancomycin
119 (VAN), using the E-test method (Oxoid) and Rifampicin (RIF) and Rifaximin (RFX)
120 resistance by broth dilution (see supplementary data) using a 2-fold antibiotic dilution range
121 from 512 - 0.5 µg/ml. *C. difficile* strains 630Δ*erm*, 630 wild type (WT) and R20291 were
122 used as controls. Guideline MIC breakpoints for RIF and RFX were taken from (7), where
123 isolates with MICs ≥32 µg/ml were considered resistant. Intermediate resistance was defined
124 as an MIC of 0.003–32 µg/mL (8).

125 **Genotypic Characterisation**

126 DNA was extracted from strains using a Phenol:Chloroform:Isoamyl alcohol (25:24:1)
127 saturated with 10 mM Tris, pH 8.0, 1 mM EDTA (Sigma) extraction method adapted from
128 (9)(See supplementary data).

129 **Whole Genome comparison**

130 Genomic DNA was sent for Illumina sequencing using MisSeq 250-PE technology,
131 (DeepSeq, University of Nottingham). DNA from one of the isolates from sample A (earliest
132 RT002 isolate), and sample E (earliest RT014 identified) designated E2 were also sent for

133 Pacific Bioscience (PacBio) Sequencing (McGill University and Genome Québec Innovation
134 Centre). Paired-end reads from the MiSeq runs were mapped to PacBio contigs using CLC
135 Genomics Workbench Version 8.0.2 (Qiagen).

136

137 **Concordance of PacBio and Illumina sequencing**

138 To demonstrate the concordance of two different sequencing methods (especially over
139 homopolymer regions), Illumina paired-end sequencing reads from isolate A and E2 were
140 mapped back to the Pacbio reference contigs. Basic variant detection (CLC Genomics
141 Workbench Version 8.0.2 [Qiagen]) was used to call single nucleotide variations (SNVs),
142 insertions and deletions. All default parameters were kept the same apart from the minimum
143 frequency setting in the coverage and count filters process. The minimum frequency setting
144 was changed to 50% to try and capture as many high quality changes as possible.

145 **Results**

146 **Ribotype of *Clostridium difficile* Isolates obtained from stool**

147 During the course of infection two distinct *C. difficile* PCR-ribotypes were isolated (Table 2).
148 The predominant PCR-ribotype occurring during the infection was RT002. Co-infection of
149 the participant with a second PCR-ribotype (RT014), was detected in stool sample E. This
150 sample was a diagnostic sample obtained after the participant had suffered the first relapse
151 (Table 2). However, it cannot be deduced whether this PCR-ribotype was present in earlier
152 samples (i.e. C or D) due to the low number of colonies obtained from the stools of these
153 samples. The RT014 isolate, may therefore, have been present at low frequency in these
154 samples.

155

156 **Isolate Growth, Sporulation and toxin quantification**

157 All isolates showed similar growth profiles in both BHIS and TY (data not shown) except
158 isolate G. This isolate had a shorter stationary phase when grown in BHIS and reduced
159 growth in TY broth. However, this did not affect sporulation and toxin titre as there were no
160 significant differences in these phenotypes, between any of the isolates (data not shown).

161 **Pacific Bioscience and Illumina MiSeq Sequencing of RT002 and RT014 isolates**

162 The PacBio sequencing was able to assemble the genome of isolate A into four contigs
163 (Table 3) and isolate E2 into one contig of 4,330,205bp. Contigs were identified by means of
164 Blast searches using the dc-megablast option against the nt database. BLAST analysis of the
165 one contig from isolate E2 suggests that it shares sequence similarity with the
166 *Peptoclostridium difficile* genome assembly CD630DERM, chromosome : 1 (LN614756.1).

167 The individual contigs were annotated using RAST (10) and used as reference strains for
168 Illumina read mapping of all isolates.

169 Over 97% of reads from seven RT002 isolates (A, B, C, D, E1, F & G) and >97% of reads
170 from two RT014 isolates (E2 and H) mapped to the reference PacBio contigs for the
171 corresponding PCR-ribotype.

172 **Concordance of PacBio and Illumina sequencing**

173 Nine SNVs were identified in all the RT002 genomes and twelve SNVs were detected when
174 Illumina MiSeq reads were mapped back to their respective PacBio contigs. All SNVs of the
175 RT002 isolates occurred in the first contig, which represented the main *C. difficile*
176 chromosome. All the SNVs from both RT002 and RT014 isolates occurred in homopolymer
177 regions of ≥ 4 nucleotides in length. PCR amplification and Sanger sequencing of all the
178 regions in isolate A and E2 that contained the SNVs confirmed that they were true SNVs.

179 Accordingly, the reference sequences A and E2 were corrected and the sequence reads from
180 the subsequent isolates (B-G) were remapped.

181 **SNV detection in RT002 isolates**

182 Basic variant detection was performed on the reads to assess whether the sequential isolates
183 contained additional changes from the original (A) isolate (Table 4). Isolate B did not contain
184 any additional SNVs. Isolate C contained one additional SNV. The A>G nucleotide change
185 did not result in an amino acid change. Isolate D and E1 both contained the same four
186 additional SNVs compared to isolate A, two of which were non-synonymous and found in
187 *oppF* and *rpoB* (Table 4). The former encodes an oligotransport-ATP binding domain, while
188 the latter encodes a DNA-directed RNA polymerase beta subunit. These two isolates did not
189 contain the SNV found in isolate C. Isolate F contained five SNVs compared to A, however,
190 these were not the same as any SNVs in previous isolates. One of the SNVs in isolate F was
191 found in the same gene (*rpoB*), but not at the same position as in isolates D and E1. Variant
192 detection in isolate G, produced 70 SNVs. Closer inspection of these SNVs revealed that 64
193 were detected at low frequency in poorly mapped regions and were probably not real. Thus,
194 this isolate had six SNV differences compared to isolate A and B (Table 4). Five SNVs were
195 in the same regions as isolate F with two of the SNVs, in a hypothetical protein and *rpoB*,
196 being in the exact same location as in isolate F. (Table 4)

197 **SNV detection in RT014 isolates**

198 Six additional SNVs were found in isolate H compared to isolate E2. Five of these SNVs
199 were in a gene annotated as *fliK*, but which is not actually part of the flagellar operon. Only
200 two of these five SNVs were non-synonymous and occurred at a frequency <52% and with an
201 average quality of <22. Closer inspection of this region revealed that the sequence quality
202 was poor most likely due to it being repetitive, suggesting that these SNVs were not likely to

203 be real. Thus, only one additional T >C SNV was identified. The SNV was in an intergenic
204 region of the genome of isolate H, at position 2562170 bp, upstream of of a gene encoding a
205 small hypothetical protein that shows similarity to a putative membrane protein.

206 **Isolate Antibiotic resistance**

207 No isolate showed resistance to MET (breakpoint considered as resistant ≥ 2 $\mu\text{g/ml}$) or VAN
208 (breakpoint considered as resistant ≥ 4 $\mu\text{g/ml}$) (data not shown). Early RT002 isolates A, B
209 and C, showed complete susceptibility to rifampicin (RIF) and rifaximin (RFX) (Figs. S1a,
210 S1b, S2a & S2b). However, RT002 isolates D (from the relapse sample) and E1 (post relapse
211 sample) showed high resistance (≥ 256 $\mu\text{g/ml}$) to RIF and RFX (≥ 128 $\mu\text{g/ml}$), whilst RT002
212 isolates F and G showed intermediate resistance to both (RIF; ≥ 4 $\mu\text{g/ml}$, RFX; ≥ 16 $\mu\text{g/ml}$).
213 The RT014 isolates (E2 and H) and control strains were fully susceptible to RIF and RFX
214 (>0.5 $\mu\text{g/ml}$). (Figs. S1a, S1b, S2a & S2b).

215 **Frequency of *rpoB* SNVs in all cultured A to G RT002 isolate samples**

216 To try and discern whether there were two distinct populations of RIF and RFX resistant
217 RT002 isolates, the region in *rpoB* that contained the SNVs identified in Table 4, was
218 amplified from every isolate with a PCR-ribotype banding pattern confirmed with the
219 QIAxcel (Table 1). The PCR amplified DNA fragment was sent for Sanger sequencing and
220 the sequences were checked for the above SNVs. The frequency of these SNVs in each
221 isolate is detailed in Table 5.

222 **Discussion**

223 The recurrent infection suffered by participant 01008 is one which is endured by up to 20%
224 of patients suffering from CDI (1). Over a 180 day period this particular individual relapsed
225 twice with CDI and presented with co-infection of two different PCR-ribotypes. At one

226 particular point during the infection the participant also, either acquired a RT002 isolate
227 which was resistant to rifamycins (re-infection), or the original isolate developed rifamycin
228 resistance, due to microevolution (relapse). The combination of these factors could have
229 contributed to the persistence of this participant's infection.

230 The nature of the RAPID trial is to recruit participants at the end of their standard CDI
231 therapy, when the patient has been assessed as being resolved of CDI. The participant is then
232 started on a regimen of either RFX or placebo for four weeks during which stool samples are
233 collected. The participant is then followed up for another eight weeks during which more
234 stool is collected. As this was a randomised double blind placebo controlled clinical research
235 trial, at the point at which this research was undertaken, it was not known if participant 01008
236 was on placebo or rifaximin therapy.

237 What is clear from this data, is that even though this participant was deemed to have resolved
238 symptoms of CDI prior to trial enrolment, there was still a low level of *C. difficile* in the stool
239 (as indicated by the isolation of *C. difficile* from sample C) and in this participant it may have
240 contributed to the recurrence of disease. There is further genetic evidence to suggest that the
241 RT002 isolate found in the pre-enrolment diagnostic specimens (A and B) had persisted, at
242 least until the next sample specimen (C). According to others (11-13) genetically identical
243 strains differ by ≤ 2 SNVs and there was only one SNV difference between isolate A/ B and
244 C. At some point during the time between sample C and sample D, participant 01008 either
245 acquired a genetically distinct RT002 isolate (re-infection) with resistance to RIF and RFX,
246 or the original RT002 isolate evolved to become RIF and RFX resistant (≥ 256 $\mu\text{g/ml}$ and
247 (≥ 128 $\mu\text{g/ml}$ respectively) relapse. Prior to sample D all isolates from samples A, B and C did
248 not contain SNVs in the *rpoB* gene (Table 5), supporting the notion that participant 01008
249 was probably colonised with one population of non-resistant RT002 isolates at that time of
250 the infection. In other studies (11, 12) genetically distinct isolates are differentiated by >10

251 SNVs. Here isolate D differed by only four SNVs compared to isolate A,B and C and within
252 the population of isolates from sample D (Table 5) one isolate did not contain the SNV in the
253 *rpoB* gene. Thus, it is not possible to deduce whether this was a newly acquired isolate or
254 whether the initial strain had mutated. It is possible that the RT002 isolate in this infection
255 was under high antibiotic selection pressure and thus may have mutated more rapidly than
256 others have calculated for strains not under intense selection pressure(14).

257 Whole genome sequencing of isolates A-H revealed that the probable cause of RIF and RFX
258 resistance in isolate D, was a C>A SNV at position 1465bp in the *rpoB* gene, encoding a
259 DNA-directed RNA polymerase beta subunit (Table 4). Mutations in *rpoB* have been
260 identified in *C. difficile* (7, 8) and also occur in multi-drug resistant strains of *Mycobacterium*
261 *tuberculosis* (MTB) (15) within a small 23 amino acid region from position 511-533 (16).
262 This suggests a common mechanism by which resistance to this antibiotic occurs in multiple
263 bacterial species.

264 The SNV in isolate D resulted in a Glutamine > Lysine amino acid change at position 489 of
265 the peptide. It is known that the Glutamine residue at the corresponding position of RpoB in
266 *Thermus aquaticus* directly binds to rifampicin (7, 17). Thus, it is likely that this SNV is
267 directly responsible for resistance to RIF and RFX in this isolate and this may have played a
268 role in the first relapse suffered by this participant. A subsequent stool specimen from
269 participant 01008 revealed the presence of a genetically identical RT002 isolate (E1) to
270 isolate D, which also shared the same SNV change in *rpoB* and was also RIF and RFX
271 resistant (≥ 256 $\mu\text{g/ml}$ and ≥ 128 $\mu\text{g/ml}$ respectively). The sample also contained another
272 ribotype (RT014 - E2). Isolate E2 was fully sensitive to RIF and RFX. If participant 01008
273 was on RFX therapy then it is unlikely that isolate E2 contributed to the relapse, as clearly the
274 vegetative form of the isolate is susceptible to the rifamycins and would have been killed.

275 However, whether or not the isolate was present just prior to relapse, is not discernible, as it
276 was only possible to isolate one colony from the pre-relapse sample C. The RT014 isolate
277 (E2) may have been present in the host at low levels in the spore form, evading the effects of
278 possible antibiotic therapy, and persisting until a time when it could germinate and grow.
279 This hypothesis is supported by the emergence of the RT014 (H) isolate in the final stool
280 sample which was genetically indistinct from isolate E2.

281 Isolates D and E1 also shared a SNV in the oligotransport-ATP binding domain of *oppF*
282 (Table 4), which belongs to an operon of oligopeptide permease (*opp*) genes that are involved
283 in regulating sporulation (amongst other processes) in some species of *Bacillus* and
284 *Clostridium* (18, 19). *In vitro* sporulation studies on all isolates revealed no significant
285 difference (data not shown) in the rate of sporulation between isolate D, E1 and all other
286 isolates. However, this data may not be representative of *in vivo* sporulation characteristics;
287 therefore it cannot be conclusively proven that this SNV had no effect. The *opp* operon is
288 involved in other processes in other organisms, for example competence in *Bacillus* and
289 *Streptococcus* species, plasmid transfer in *Enterococcus faecalis*, and the expression of
290 virulence factors in *Bacillus thuringiensis* (18, 20). SNVs in this region may, therefore, have
291 a yet undiscovered role in *C. difficile* virulence and could present a further avenue of
292 research.

293 The next specimen provided by the patient revealed the presence of an RT002 isolate (F) with
294 five additional SNVs compared to isolate A, B and C. Four of the SNVs were completely
295 different to the previous isolate (E1) and isolate D. However, one SNV was again located in
296 *rpoB*, at a different position (1475bp), resulting in an A>T change that caused an amino acid
297 change from aspartic acid > valine at position 492 of the peptide. The substitution of this
298 aspartic acid residue to other amino acids, including valine, has been shown to result in RIF
299 resistance in *Staphylococcus aureus* (7, 21). Unlike the RpoB amino acid changes in isolates

300 D and E1, the RpoB amino acid change in isolate F, apparently conferred only intermediate
301 resistance to RIF($\geq 4 \mu\text{g/ml}$) and RFX ($\geq \mu\text{g/ml}$). The SNV at this position was also shared
302 by the final RT002 isolate found in sample G, which showed the same level of resistance to
303 RIF and RFX. Whether or not this level of resistance would be sufficient to contribute to the
304 persistence of the organism in the colon is unclear, but there is clearly selective pressure for
305 this mutation occurring either in the host or within the *C. difficile* population found at the
306 QMC in Nottingham.

307 The presence of a SNV in the same gene (*rpoB*), but at different positions within the RT002
308 isolates, supports the notion that two distinct subpopulations of RIF and RFX resistant RT002
309 isolates could have co-existed in this participant. Further sequencing of this region in sample
310 E RT002 isolates, revealed that they all shared the same C>A SNV. However, one of the
311 fifteen sample F isolates cultured (Table 5) contained the C>A SNV found in sample E
312 isolates and not the A>T SNV found in the remaining F isolates, indicating that two sub-
313 populations of RT002 isolates could have potentially co-existed at this point. Amongst the
314 sample G isolates, there were equal population of isolates with and without any SNVs in
315 *rpoB*. This either indicates the persistence of the initial susceptible isolate in the gut, or that at
316 the time sample G was taken the population was in a transient state of mutation.

317 It is poignant to note that other studies have documented cases of *C. difficile* rifamycin
318 resistance after chaser therapies using rifaximin (8, 22, 23). One of these studies has linked
319 this resistance to mutations in RpoB (8) that have been identified by others (7). The study by
320 Curry *et al.* (8) indicates that in their study population, more than one-third of isolates were
321 resistant to rifaximin and this is something which could be looked at in a wider population of
322 participants on the RAPID trial, as it may prompt clinicians to alter dosing regimen if the
323 therapy was approved for use in patients suffering recurrence.

324 Conclusion

325 This case study has presented insight in to the course of recurrent infection caused by *C.*
326 *difficile*. In this case it was difficult to ascertain whether, in this particular individual, the *C.*
327 *difficile* strain was evolving. However, it did reveal the possible presence of multiple isolates
328 with SNVs causing distinct fitness advantages. The fact that this participant was enrolled in a
329 trial to investigate the use of RFX to prevent recurrence suggests that we should be
330 monitoring mutations in the *rpoB* gene more closely in the isolates from trial patients, as a
331 side effect of this therapy could be the increased selection for RFX resistant *C. difficile*
332 strains. This is of importance to clinicians as it may directly impact the antibiotic regimen
333 they use to treat their patient.

334 The advent of high throughput technologies will allow for more in depth screening of
335 samples to elucidate the true genetic fingerprint of the isolates found during infection. When
336 coupled with in-depth microbiome analysis of the host, this may allow researchers to more
337 fully comprehend the overall picture of recurrent infection and in turn this translate this
338 information to clinicians, in order to manage 'at risk' patients more effectively and reduce the
339 morbidity and economic burden of *C. difficile* within the healthcare system.

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345 Conflict of Interest

346 The authors declare that there is no conflict of interest

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448 **Table 1-** Description and date of sample collection for participant 01008 on the RAPID trial

ID	Specimen	Sample collection date
A	1 st diagnostic sample*	21/6/13
B	2 nd diagnostic sample*	1/7/13
C	Week 0 (visit 1) RAPID sample	9/7/13
D	Relapse sample 1	19/7/13
E	Diagnostic sample post relapse sample 1*	27/7/13

F	Week 4 (visit 3) RAPID sample	7/8/13
G	Week 12 (Visit 5) RAPID sample	2/10/13
H	Relapse sample after week 12 (visit 5) RAPID sample	4/1/14

*Samples obtained from Queens Medical Centre microbiology department

Table 2- PCR-ribotype of isolates obtained from participant 01008 stools samples

ID	Specimen	Sample collection date	Number of Colonies obtained/typed*	PCR-ribotype of isolate
A	1st diagnostic sample	21/6/13	20/8	002
B	2 nd diagnostic sample	1/7/13	20/20	002
C	Week 0 (visit 1) RAPID sample	9/7/13	1/1	002
D	Relapse sample 1	19/7/13	8/5	002
E	Diagnostic sample post relapse sample 1	27/7/13	20/20	E1-002 (7) E2- 014 (13)
F	Week 4 (visit 3) RAPID sample	7/8/13	15/14	002

G	Week 12 (Visit 5) RAPID sample	2/10/13	20/20	002
H	Relapse sample after week 12 (visit 5) RAPID sample	4/1/14	20/20	014

*In house capillary electrophoresis typing using a QIAxcel. Numbers in brackets of column five represent the frequency of that PCR-ribotype pattern

Table 3: PacBio Contig Assembly information from RT002 isolate A.

Contig	PacBio BLAST identification	Contig Size
1	FN668944.1, <i>Clostridium difficile</i> BI9 chromosome	4,207,942bp
2	LN681537.1, <i>Clostridium</i> phage phiCD211, complete genome	140,450bp
3	FN668942.1, <i>Clostridium difficile</i> BI1 plasmid pCDBI1, complete sequence	65,380bp
4	GU949551.1, <i>Clostridium</i> phage phiCD6356, complete genome	52,160bp

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Strain	Reference Position	Type	Reference	Allele	Count	Coverage	Frequency	Average quality	Coding region change	Amino acid change
C	319864	SNV	A	G	194	201	96.52	33.44	Choline binding protein A:c.6498A>G	
D	147783	SNV	A	T	189	192	98.44	36.37	Aminobenzoyl-glutamate transport protein:c.210T>A	
D	556338	Deletion	A	-	177	177	100.00	31.07	Oligopeptide transport ATP-binding protein OppF (TC 3.A.1.5.1):c.479delA	p.Gln160fs
D	2974068	SNV	G	A	210	233	90.13	36.21		
D	3762293	SNV	C	A	189	191	98.95	36.61	DNA-directed RNA polymerase beta subunit (EC 2.7.7.6):c.1465C>A	p.Gln489Lys
E1	147783	SNV	A	T	195	198	98.48	34.89	Aminobenzoyl-glutamate transport protein:c.210T>A	
E1	556338	Deletion	A	-	146	149	97.99	32.74	Oligopeptide transport ATP-binding protein OppF (TC 3.A.1.5.1):c.479delA	p.Gln160fs
E1	2974068	SNV	G	A	151	211	71.56	36.64		
E1	3762293	SNV	C	A	161	161	100.00	36.15	DNA-directed RNA polymerase beta subunit (EC 2.7.7.6):c.1465C>A	p.Gln489Lys
F	1181867	SNV	G	T	172	174	98.85	33.01	FIG00512976: hypothetical protein:c.722C>A	p.Thr241Asn
F	1861424	SNV	A	G	158	168	94.05	31.75		
F	1861431	SNV	G	A	171	172	99.42	35.88		
F	2676955	SNV	A	C	231	233	99.14	35.90		
F	3762308	SNV	A	T	204	207	98.55	34.08	DNA-directed RNA polymerase beta subunit (EC 2.7.7.6):c.1475A>T	p.Asp492Val

G	1181870	SNV	G	T	33	33	100	37.12	FIG00512976: hypothetical protein:c.722C>A	p.Thr24 1Asn
G	1861429	SNV	A	G	21	21	100	32.95		
G	1861436	SNV	G	A	22	22	100	37.64		
G	2676961	SNV	A	C	12	12	100	38.67		
G	3762308	SNV	A	T	143	174	82.18	36.96	DNA-directed RNA polymerase beta subunit (EC 2.7.7.6):c.1475A>T	p.Asp4 92Val
G	3040149	SNV	C	A	21	33	63.64	38.24	FIG00534171: hypothetical protein:c.13G>T	p.Glu5*

Table 4- Additional SNVs in genome of sequential RT002 isolates

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Table 5. Frequency of all cultured RT002 isolates with and without *rpoB* SNVs

ID	Specimen	Number of Colonies typed	SNV present*
A	1st diagnostic sample	8	-
B	2nd diagnostic sample	20	-
C	Week 0 (visit 1) RAPID sample	1	-
D	Relapse sample 1	5	+ (4) [C>A] - (1)
E	Diagnostic sample post relapse sample 1	7	+
F	Week 4 (visit 3) RAPID sample	14	+ [A>T (13)] + [C>A (1)]
G	Week 12 (Visit 5) RAPID sample	20	+ (10) [A>T] - (10)

* (-): absence of SNV, (+): presence of SNV. Numbers in brackets are the frequency of the isolates with or with the SNV. Square brackets identify which SNV was present.

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503 **Figure 1-** A simplified study timeline indicating the collection of stool samples for the participants involved in
504 the RAPID trial.

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